Ocular Irritation Protocol: Sub-Draize Mildness Testing (MTT ET-50)

For Use with EpiOcular™ Tissue Model (OCL-200)

Overview: This protocol involves applying neat test articles to the EpiOcular tissue model and is used to differentiate between materials for which the standard Rabbit Eye Draize testing is insensitive.

The MTT tissue viability assay is utilized to determine the time of exposure needed for a test article to reduce the viability to 50% of control tissues (ET-50). Based on the ET-50 and a comparison to benchmark materials/formulations, the degree of mildness can be estimated.

This protocol is identical to the method contained within the "Ocular Irritation Protocol – Neat Testing" except that exposure times are extended well beyond the 1-hour maximum exposure time therein.

I. Storage of EpiOcular (OCL-200) and MTT Kit (MTT-100) Components

a) **Storage:** Upon receipt of the EpiOcular Tissue Model, place the sealed 24-well plate containing the EpiOcular tissues and the assay medium into the refrigerator (2-8°C). If you have ordered the MTT toxicology kit (part # MTT-100) or the MTT diluent solution (part # MTT-100-DIL), place the MTT concentrate containing vial in the freezer (-20±5°C) and the MTT diluent in the refrigerator. Storage conditions are summarized in the following table.

Part #	Description	Conditions	Shelf Life
OCL-200	EpiOcular tissues	Refrigerate (2-8°C)	96 hours*
OCL-200-ASY	Assay Medium	Refrigerate (2-8°C)	3 weeks**
MTT-100-DIL***	MTT diluent	Refrigerate (2-8°C)	2 months**
MTT-100-CON***	MTT concentrate	Freezer (-20±5°C)	2 months**

*Note: *Refers to storage time @ 2-8°C in unopened package.*
***From date of manufacture. See component label for the expiration date.*
****MTT kits must be ordered separately.*

II. Preparation of EpiOcular

a) **Pre-warm media:** Pre-warm the MatTek assay medium (provided) to 37°C. Using sterile technique, pipet 0.9 mL of the assay medium into each well of the sterile 6-well plates (provided). Label the 6-well plates indicating the test material and the dosing time to be used.

b) **Transfer EpiOcular tissues:** 1 hour before dosing is to begin, remove the EpiOcular tissue from the refrigerator. Under sterile conditions using sterile forceps, transfer the EpiOcular tissues into the 6-well plates containing the pre-warmed assay medium. *Note: Care should be taken to remove all adherent agarose sticking to the outside of the cell culture inserts containing the EpiOcular tissue.*

c) **Partial kit testing:** If all 24 tissues are not needed on day 1 of testing or if starting times for dosing differ by more than 1 hour, carefully open the plastic bag containing the 24-well plate/EpiOcular tissues and remove the tissues for day 1 testing under sterile conditions as per the previous step. Return the cover to the 24-well plate containing the remaining tissues and put the 24-well plate back in the

original bag without sealing it (a new plastic bag, which can be later sealed, can also be used). Place the 24-well plate in the open plastic bag into the incubator at 37°C and 5% CO_2. Allow the atmosphere within the bag to re-equilibrate with 5% CO_2 for 10 minutes. Prior to removing the bag from the incubator, re-seal the bag using tape so that the 5% CO_2 atmosphere will be maintained. Return the sealed bag to the refrigerator (2-8°C) where it can be stored for an additional 24 hours.

d) **Incubate:** Place the 6-well plates containing the EpiOcular tissues into a humidified 37°C, 5% CO_2 incubator for 1 hour prior to dosing.

III. Dosing

a) **Exposure times:** Exposure times are 1, 5, and 24 hours. Test articles are applied to triplicate EpiOcular tissues. Duplicate tissues can be used once the user has experience using the EpiOcular tissue protocols. *Note: For scheduling convenience, if necessary, a 4-hour dose can be used in lieu of the 5-hour dose.*

b) **Replace assay medium:** Following the 1 hour incubation, aspirate off the assay media contained within the 6-well plates and replace with 0.9 mL (per well) of pre-warmed, fresh assay media. *Note: Any air bubbles trapped underneath the cell culture insert should be released (tilt the cell culture insert with a sterile forceps) so that adequate nutrients are supplied to the EpiOcular tissues during the dosing period.*

c) **Negative controls:** 3 (or 2) tissues are dosed with 100 µL of deionized or ultrapure water to serve as a negative control. All manipulations with these negative controls should be identical to those inserts which are exposed to test materials. Exposure time for the negative controls is 5 hours.

d) **Apply dose:** Pipet 100 µL of test material into the cell culture insert atop the EpiOcular tissue. Viscous materials should be pipetted with a positive displacement pipette. Do not add the test material to the assay medium in the well. Negative controls should be treated in an identical manner to the dosed inserts. See Figure 1. Return the 6-well plates containing the dosed EpiOcular tissues to the incubator for the desired time period.

e) **Non-pipettable materials**: For materials that cannot be pipetted, applicator pins should be used to provide a reproducible, even means of application. For solids, a 1:1 slurry/paste of material and ultrapure H_2O is made and 200 mg of the slurry is applied using the applicator pin. *Note: Different solid/H_2O ratios can be used to improve the consistency of the slurry/paste, however, the amount of slurry applied to the applicator pin should be adjusted so that 100 mg of the original material are applied.* Stick the pin into a small piece of Styrofoam, tare it on an analytical balance, and apply the appropriate amount of slurry/paste to the flat end of the pin. Gently place the applicator pin onto the tissue surface in the bottom of the tissue culture insert to bring the test article into contact with the tissue.

f) **Prepare MTT solution:** Prior to the end of the first dosing period, prepare the MTT solution. If you are using the MatTek MTT toxicology kit (Part # MTT-100), thaw the MTT concentrate and dilute with the MTT diluent (provided). If you are making your own MTT solution, use 1 mg/mL MTT diluted in DMEM. Spin down (300 g for 5 minutes) or filter the MTT solution to remove any precipitate. Store the remaining MTT solution in the dark at 4°C for the later time points. *Note: To obtain optimal results, MTT solutions should not be stored for more than 1 day since MTT will degrade with time.*

g) **Reduction of MTT by Test Article:** To insure that the MTT reaction is accurately measuring the tissue viability, it is necessary to determine whether the test article (TA) can directly reduce MTT. A 1.0 mg/mL MTT solution is prepared as above and 100 µL or 100 mg of the neat TA is added to 1 mL of the MTT solution. This mixture is placed in the incubator 37°C, 5% CO_2 for 24 hours. A negative control, 100 µL of ultrapure water, is tested concurrently. If the MTT solution color turns blue/purple, the TA has reduced the MTT; the absence of darkening indicates that the TA does not directly reduce MTT.

If the TA does reduce MTT, a false viability measurement is obtained only if the TA remains bound to the tissue after rinsing. To test for residual TA induced MTT reduction, a single killed tissue (part # OCL-200-Frozen) is treated with the TA in the normal fashion. The incubation should be the longest incubation used for that TA. Rinsing and MTT exposure should be performed normally. An untreated killed control will be tested in parallel since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue. If little or no MTT reduction is observed in the TA treated killed control, the MTT reduction observed in the TA treated viable tissue may be ascribed to the viable cells. If there is appreciable MTT reduction in the treated killed control (relative to the amount in the treated viable tissue), additional steps must be taken to account for the chemical reduction or the TA may be considered untestable with MTT. If the direct reduction of MTT by the TA is less than 30% of the negative control value, the net OD of the treated killed control may be subtracted from the new ODs of the live TA-treated tissues. If the direct reduction by the TA is greater than 30% of the negative control value, please contact MatTek technical assistance for further guidance.

h) **Prepare MTT plate:** Prepare a 24-well plate (provided) w/ MTT solution by pipetting 300 µL of the MTT solution into the appropriate number of wells of the 24-well plate to accommodate all the inserts for the time period which is ending. Label the 24-well plate top to indicate to which wells the tissues will be transferred. Label the second 24-well plate in an identical manner for later use in the extraction step. Also, label vials for media samples storage if LDH or inflammatory mediator release measurements are to be made.

i) **Removal of test material:** After exposure of the EpiOcular tissues to the test materials is complete, discard any liquid remaining atop the EpiOcular tissues. For non-flowing, viscous materials, the sample can often be removed by drawing it off with a disposable Pasteur pipette. Next submerge each insert individually to fill it with PBS and then decant. Repeat this procedure 3 times or as many times as necessary to remove any residual test material. Following the 3 rinses submerge the cell culture insert containing tissue in 5.0 mL of assay media for 10 minutes at 37ºC and 5%CO_2. See Figure 2. This final soak removes any residual absorbed test material and is crucial to obtaining accurate, repeatable dose response curves.

j) **Transfer tissues to MTT plate:** After the 10 minute soak, decant and shake off the assay medium prior to placing the EpiOcular tissue in the MTT containing 24-well plate. When the cell culture inserts have been transferred to the MTT plate, make sure that no air bubbles are trapped underneath the cell culture insert.

k) **Media for inflammatory mediator analysis:** If cytokine release will be measured, save the assay media from the 6-well plates in labeled vials for subsequent analysis. LDH, PGE-2, IL-1α and other inflammatory mediators/cytokines can be measured. For LDH, test immediately or store samples at 4°C for up to 24 hours; for IL-1α, store at 2-8°C for up to 7 days; for PGE-2, store samples under nitrogen and freeze.

l) **MTT loading:** Return the EpiOcular tissues in the 24-well plate to the incubator for 3 hours. See Figure 3. Note: Deviations from the 3-hour time for MTT incubation will result in different MTT readings and thus the 3-hour MTT incubation time should be adhered to very strictly (3.0 hours ± 5 minutes or less is recommended).

IV. Extraction

a) **Transfer tissue to extraction plate:** After the 3 hour MTT incubation period is complete, remove each insert individually and gently rinse with PBS to remove any residual MTT solution. Remove excess PBS by gently shaking the insert and then blotting the bottom with a Kimwipe. Finally, place the inserts into the pre-labeled 24 well extraction plate.

b) **Add extractant:** Immerse the inserts using 2.0 mL of extractant solution per well, completely covering the EpiOcular tissue. See Figure 4. Cover the extraction plate to reduce evaporation of extractant. *Note: If the test article is colored and/or does not completely rinse off the tissue, pipet 1.0 mL into the well so that the MTT is extracted through the bottom of the tissue culture insert*. After extraction is complete, remove the insert and add an additional 1.0 mL of extractant to bring the total volume up to 2.0 mL.

c) **Extraction conditions:** Place the extraction plate with its top in place in a sealable plastic bag (e.g. Zip-lock) to minimize extractant evaporation. Allow the extraction to proceed for 2 hours at room temperature (RT) on an orbital shaker or overnight (no shaking) at RT in the dark. Protect plate from light while shaking with aluminum foil. Shaking should be vigorous enough for some mixing within the wells, but not fast enough that liquid will leave the wells. Note: It is often most convenient to allow the extractions to proceed until all samples are complete so that all MTT readings can be made at once. As long as evaporation of solvent is prevented, extraction times beyond 2 hours will not affect MTT readings. If you are using your own reagents, the extractant should **not** contain acid (e.g. hydrochloric acid).

d) **Decant extractant in 24-well plate:** After the extraction period, decant the liquid within each insert back into the well from which it was taken (i.e. mix the solution with the extractant in the well). The inserts can be discarded.

V. Determine ET-50

a) **Mix extractant solutions:** Pipet the extractant solution up and down at least 3 times to insure that the extraction solutions are well mixed.

b) **Transfer to 96-well plate:** Pipet 200 μL of the mixed extraction solution into a 96 well microtiter plate. Note: if a 96-well plate reader is not available, a spectrophotometer can be used to determine optical density of the extractant solution.

c) **Measure optical density:** Determine the optical density of the extracted samples at a single wavelength between 540 and 570 nm using 200 μL of the extractant as a blank.

d) **Calculate % viability:** Determine % viability at each exposure time using the following formula:

% Viability = 100 x [OD(treated tissue)/OD(negative control)

e) **Determine ET-50:** Using a semi-log scale, plot the % viability (linear y axis) versus the dosing time (log x axis). By interpolation, the time at which the % viability has dropped to 50% is considered the ET-50 value. See Figure 5. Note: Interpolation for the ET-50 is best done mathematically. An Excel spreadsheet to facilitate ET-50 calculations is available from MatTek Corporation.

f) **Positive control:** 0.3% Triton X-100 is provided with the kit. The typical ET-50 falls between 15 and 45 minutes. See Figure 5. Recommended dosing times are 15 and 45 minutes along with 4 minutes if enough tissues are available. Alternatively, MatTek tests every lot of EpiOcular with 0.3% Triton X-100 – these data are available by Wednesday each week.

VI. Correlation of In Vitro and In Vivo Results

a) **Sample ET-50 data:** Based on a publication by Kay, J.H. and Calandra, J.C. ("Interpretation of eye irritation tests", J. Soc. Cosmetic Chem., 13, 281-289, 1962), Draize scores below 15 are classified as "minimal" to "non-irritating". However, for eye care products or cosmetics used in the vicinity of the eyes (where some inadvertent exposure will inevitably occur), mildness levels, below the point at which the Draize test can differentiate, are often required. As shown in Table 1, the OCL-200 can be used to differentiate between low levels of surfactants for which the Draize test is insensitive.

Benzalkonium Chloride (BAK)		
In Vivo Concentration	**Draize (MMAS)**	**ET-50 (min)**
0.10%	0	212.7
0.03%	0	2053.0

Sodium Dodecyl Sulfate (SDS)		
In Vivo Concentration	**Draize (MMAS)**	**ET-50 (min)**
0.30%	0	740.1
0.10%	0	1938.3

Table 1: Use of EpiOcular to differentiate between very mild materials that cannot be distinguished by the in vivo Draize test.

b) **Developing your own correlation – use of Benchmark materials:** In order to distinguish between levels of mildness or ultra-mildness, it is necessary to establish ET-50 values for materials of known clinical/end-use irritancy. For instance, ET-50's should be determined for: a) formulations on the market known to be acceptable in terms of mildness and b) formulations known to be unacceptable in terms of ocular irritancy. These benchmark ET-50's can then be used to evaluate the ocular irritancy of new formulations. Longer ET-50's indicate an expected increase in mildness, whereas shorter ET-50's indicate an increase in expected ocular irritancy.

c) **Published benchmark data:** Benchmark data for a broad range of cosmetic products is contained in the poster: "Assessment of Ocular Irritation Ranges of Market-Leading Cosmetic and Personal-Care Products Using an In Vitro Tissue Equivalent," N.E. McCain, R.R. Binetti, S.D. Gettings, and B.C. Jones, Avon Products, Inc. Presented at 2002 Soc. of Toxicology Meeting. Contact MatTek Technical Service for a copy of the poster.

d) **Significance of differences in ET-50:** In order to evaluate the significance of differences in ET-50's between materials, it is important to take into account the absolute ET-50 value. For instance, ET-50's of 2.0 and 3.5 hours probably represent real, clinically discernible differences in ocular irritancy. However, differences in ocular mildness for materials with ET-50's of 22.0 and 23.5 may not be distinguishable. MatTek scientists are always willing to assist you in interpreting your experimental results.

VII. Materials Provided

EpiOcular (Part No. OCL-200)

Quantity	Description	Part No.
24	EpiOcular tissues	OCL-200
4	6-well plates (sterile)	MW-15-003-0027
2	12-well plates (sterile	MW-15-003-0030
2	24-well plates (sterile)	MW-15-003-0028
100 mL	PBS rinse solution	TC-PBS
200 mL	Assay medium	OCL-200-ASY
10 mL	0.3% Triton X-100 solution	TC-TRI-0.3%
1	MTT protocol	MK-24-007-0031

VIII. Optional Materials

MTT Assay Kit (Part No. MTT-100)

Quantity	Description	Part No.
8 mL	MTT diluent solution	MTT-100-DIL
60 mL	Extractant solution	MTT-100-EXT
2 mL	MTT concentrate (5:1)	MTT-100-CON

Additional Materials

Quantity	Description	Part No.
24	Applicator pins	EPI-PIN-24

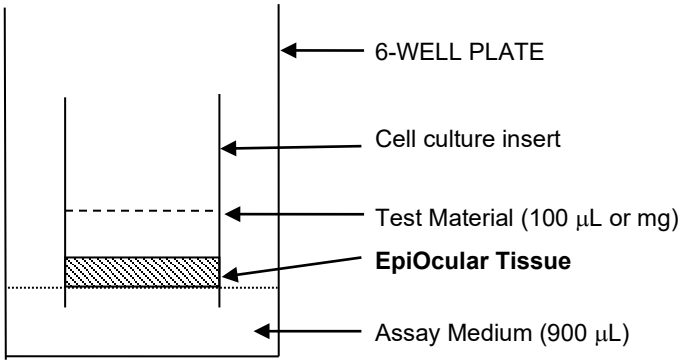

Figure 1: Dosing (37° C, 95% rH, 5% CO_2)

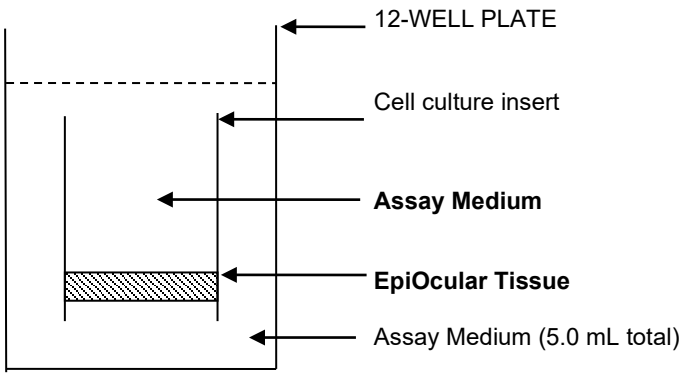

Figure 2: Soaking (37° C, 95% rH, 5% CO_2, for 10 min)

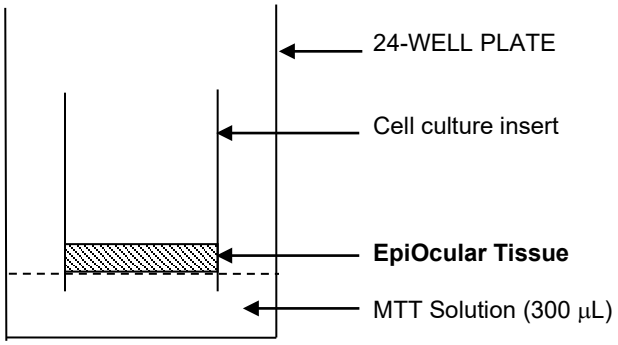

Figure 3: Incubation in MTT (37° C, 95% rH, 3 hours)

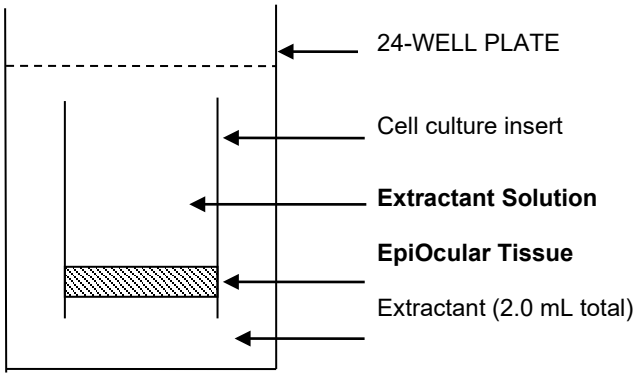

Figure 4: Extraction (Room temp., in the dark)

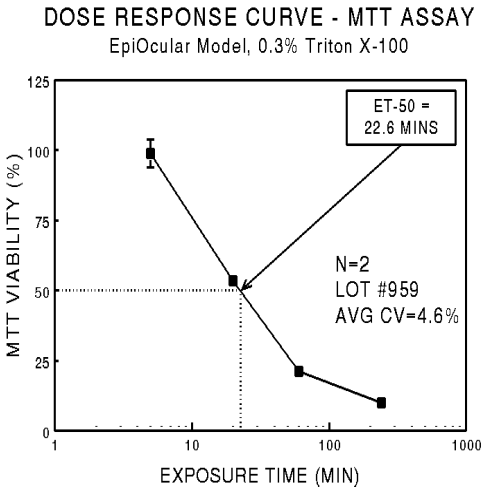

Figure 5: Typical dose response curve for EpiOcular tissues following exposure to the positive control, 0.3% Triton X-100.